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Ca^{2+} Transients Are Not Required as Signals for Long-term Neurite Outgrowth from Cultured Sympathetic Neurons

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Abstract. A method for clamping cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) in cultures of rat sympathetic neurons at or below resting levels for several days was devised to determine whether Ca^{2+} signals are required for neurite outgrowth from neurons that depend on Nerve Growth Factor (NGF) for their growth and survival. To control $[Ca^{2+}]_i$, normal Ca^{2+} influx was eliminated by titration of extracellular Ca^{2+} with EGTA and reinstated through voltage-sensitive Ca^{2+} channels. The rate of neurite outgrowth and the number of neurites thus became dependent on the extent of depolarization by KCl, and withdrawal of KCl caused an immediate cessation of growth. Neurite outgrowth was completely blocked by the L type Ca^{2+} channel antagonists nifedipine, nitrendipine, D600, or diltiazem at sub- or micromolar concentrations. Measurement of $[Ca^{2+}]_i$ in cell bodies using the fluorescent Ca^{2+} indicator fura-2 established that optimal growth, similar to that seen in normal medium, was obtained when $[Ca^{2+}]_i$ was clamped at resting levels. These levels of $[Ca^{2+}]_i$ were set by serum, which elevated $[Ca^{2+}]_i$ by ~30 nM, whereas the addition of NGF had no effect on $[Ca^{2+}]_i$. The reduction of $[Ca^{2+}]_o$ prevented neurite fasciculation but this had no effect on the rate of neurite elongation or on the number of extending neurites. These results show that neurite outgrowth from NGF-dependent neurons occurs over long periods in the complete absence of Ca^{2+} signals, suggesting that Ca^{2+} signals are not necessary for operating the basic machinery of neurite outgrowth.

NEURITE outgrowth is a complex process where signals that instruct the production of neurites must be coordinated with signals that drive the underlying cellular machinery. The biochemical mechanisms that are responsible for coordinating neurite outgrowth are not yet completely understood (9, 35), but Ca^{2+} signals are suggested to play a major role in this process (9, 21). In early studies that focused on growth cone motility, participation of Ca^{2+} was inferred from correlations between growth cone behavior and extracellular treatments, giving rise to some confusion as to whether growth cone motility increases or decreases with elevation of $[Ca^{2+}]_i$ (for example, 2, 6, 14, 27). Direct measurements of $[Ca^{2+}]_i$ using Ca^{2+} indicators such as fura-2 showed mostly increases in $[Ca^{2+}]_i$ in motile growth cones (13, 15, 28). However, recent measurements of $[Ca^{2+}]_i$ in growth cones of some *Helisoma* neurons suggest that each cell may have an optimal concentration range of Ca^{2+} at which its growth cones are motile (13). Thus, elevation of $[Ca^{2+}]_i$ may promote or arrest growth cone movement depending on the basal level of $[Ca^{2+}]_i$ or "Ca^{2+} set point" (32). It is not known whether growth cones will continue to function if $[Ca^{2+}]_i$ is clamped at the permissive level for long periods.

Although growth cones play an important role in the control of pathway guidance (3, 9, 29) and are proposed to exert tension that may be transduced into signals for neurite elongation (8, 24), the reduction of filopodia and lamellipodia movements in growth cones by treatment with actin-disrupting agents does not block neurite outgrowth in vivo or in vitro (5, 30). Less is understood about the role of Ca^{2+} in the control of neurite elongation. Mattson and Kater (32) have suggested that neurite elongation and growth cone motility are dependent on different concentrations of $[Ca^{2+}]_i$, but in these experiments $[Ca^{2+}]_i$ was not measured directly.

Most of the experiments measuring $[Ca^{2+}]_i$ in neurites and growth cones with fura-2 have followed neurite outgrowth up to a few hours. Thus, it is not yet clear to what extent changes in intracellular Ca^{2+} are necessary for driving neurite outgrowth over longer periods. The interpretation of experiments aiming to study the role of Ca^{2+} in long-term outgrowth can be complicated because of the need for a viable cell body (48). In many cells, the rate of protein synthesis, for example, is highly dependent on intracellular Ca^{2+} (10, 11). In sympathetic neurons in particular, long-term survival also requires the presence of nerve growth factor (NGF).[1] In some PC12 cells, NGF caused rapid elevation of Ca^{2+} (36) while in others it enhanced the elevation of Ca^{2+} elicited by bradykinin (47). This suggests that Ca^{2+} may be used as a signal to mediate the effects of NGF; however, it

1. *Abbreviations used in this paper*: BAPTA, 1,2 *bis*(2-aminophenoxy) ethane *N,N,N',N'* tetraacetic acid; DHP, 1,4-dihydropyridines; NA, noradrenaline; N-CAM, neural cell adhesion molecule; NGF, nerve growth factor; VSCC, voltage-sensitive Ca^{2+} channels.

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is not known whether NGF evokes any changes in Ca^{2+} in neurons.

One way to test whether long-term neurite outgrowth from NGF-dependent neurons requires the production of $[Ca^{2+}]_i$ transients is to abolish the ability of neurons to generate such $[Ca^{2+}]_i$ signals. For this purpose, we clamped $[Ca^{2+}]_i$ at the minimal levels required to sustain protein synthesis by activating voltage-sensitive Ca^{2+} channels (VSCC) in cells grown in a highly buffered low Ca^{2+} medium. This study describes the application of this method to study neurite outgrowth from sympathetic neurons over several days.

Materials and Methods

Explant and Cell Culture

Explants were prepared from superior cervical ganglia of 1-d postnatal rats and cultured on coverslips coated with rat tail collagen in enriched L15-CO_2 medium (Gibco Limited, Paisley, Scotland) (17, 44, 45) with the concentrations of rat serum and 2.5*S* NGF reduced to 3% and 20 ng/ml, respectively. Medium contained 10 μM uridine, 5-fluoro-deoxyuridine, and cytosine arabinoside to eliminate nonneural cells. To isolate single cells, desheathed ganglia were incubated for 30 min at 37°C in medium (pH 7.4) containing 0.35 mM EDTA, 124 mM NaCl, 3.4 mM KCl, 10 mM phosphate buffer, 25 mM Tris base, and 0.1% trypsin (1:250; Difco Laboratories Inc., Detroit, MI). Ganglia were spun at 500 rpm for 5 min to remove trypsin, suspended in 1 ml plating medium containing 1 μg/ml DNase I (Sigma Chemical Co., Poole, England) and 5% rat serum and triturated in a fire-polished pasteur pipette (0.5-mm aperture) until a suspension of single cells was obtained. Cells were respun, suspended in plating medium, and preplated for 3 h in Nunc (Roskilde, Denmark) tissue culture dishes (6 cm diameter) to reduce the number of nonneural cells. Neurons were dislodged, respun, and plated as previously described (44). Media containing EGTA were carefully controlled for pH.

Measurement of $[Ca^{2+}]_i$

Isolated neurons were loaded with fura-2 (16) at 30°C for 30 min in L15-Hepes medium (pH 7.4) containing 10 mM Hepes, 0.6% glucose, and 4 μg/ml fura-2 acetoxymethyl ester (Molecular Probes Inc., Junction City, OR) diluted from a stock solution (1 mg/ml) containing 20% pluronic F-127. Cells were washed extensively in L15-Hepes and mounted on a heated stage of an epifluorescence microscope (Diaphot; Nikon Inc., Garden City, NY) in L15-CO_2 growth medium containing 5 mM $NaHCO_3$ and no serum or NGF. $[Ca^{2+}]_i$ was measured in cell bodies and neurites as indicated using a Deltascan system with dual excitation and single emission (20). Excitation at 340 or 380 nm changed every 25 ms, and emission was collected at 511 nm with a photomultiplier tube. The plots show 10 or 20 points collected per second. $[Ca^{2+}]_i$ was approximated by measuring fluorescence of a solution containing 0.25 and 0.5 μM fura-2–free acid and either 1 mM EGTA (pH 7.4) or 10 mM free $[Ca^{2+}]$ (pH 7.4), using the equation of Grynkiewicz et al. (16). Calibration of fluorescence ratios by titration of 50 μM fura-2 in the presence of 1 mM 1,2 *bis*(2-aminophenoxy) ethane *N,N,N',N'* tetra-acetic acid (BAPTA) and known $[Ca^{2+}]$ (similar to Thayer et al. [42]) established that resting $[Ca^{2+}]_i$ in the absence of serum was 43 ± 16 nM (n = 29).

[^{3}H]Noradrenaline (NA) Uptake and Release

l-[7,8^{3}H]NA (15 Ci/mmol in 2% acetic acid, >97% pure by TLC, Amersham International, Amersham, UK) was diluted to final concentrations of 0.34–2.2 μM in L15-CO_2 or L15-air medium and added to explants or single cell cultures as indicated. After the indicated time, cultures were washed three times for 15 min in 2.5 ml L15-air medium at room temperature, and cells on coverslips, or cell extracts in 10% aqueous acetic acid (300 μl), were either put directly into scintillation vials containing 1 ml 0.1 M HCl and 3.5 ml scintillant or a release of [^{3}H]NA was evoked as described previously (45).

Results

Establishing a Clamp on $[Ca^{2+}]_i$

The initial aim of these experiments was to set $[Ca^{2+}]_i$ at low constant levels for several days. In normal medium (containing 1.3 mM Ca^{2+}), Ca^{2+} influx was very rapid and it was difficult to obtain precise control over $[Ca^{2+}]_i$ by adding polycationic Ca^{2+} antagonists (32) without affecting neuronal viability. Organic Ca^{2+} channel blockers like methoxyverapamil (48) were ineffective in controlling Ca^{2+} influx. The strategy that was used was therefore to make Ca^{2+} influx through open Ca^{2+} channels the rate-limiting step in the supply of intracellular Ca^{2+}. By reducing $[Ca^{2+}]_o$ with increasing concentrations of EGTA (or BAPTA), the inward Ca^{2+} gradient was reduced until activation of Ca^{2+} influx became essential. Since rat sympathetic neurons contain both N and L type VSCC (18, 38, 46), we examined whether these could not be activated by depolarization in the EGTA medium and used by the cells as natural Ca^{2+} ionophores to restore sufficient Ca^{2+} influx for long-term growth.

The effect on neurite outgrowth of decreasing $[Ca^{2+}]_o$ in the presence of normal and depolarizing media is shown in Fig. 1. In normal KCl (5.4 mM or LK^+; *upper row*), neurite elongation proceeded largely unchanged when $[Ca^{2+}]_o$ was reduced ninefold from 1.3 mM (HCa^{2+}) to 150 μM Ca^{2+} by adding EGTA (Fig. 1, *A–C*), but in medium containing 50 μM Ca^{2+} (LCa^{2+}) neurite outgrowth was no longer sustained (Fig. 1 *D*). Addition of 50 mM KCl (HK^+) to this medium, however, completely restored neurite outgrowth (Fig. 1 *H*). Further examination of the dependence of neurite outgrowth on $[Ca^{2+}]_o$ and HK^+ showed that outgrowth could still be induced in 55 mM KCl when $[Ca^{2+}]_o$ was reduced to 20–25 μM, but at 10–15 μM no outgrowth was observed despite a 200-fold ratio of $[Ca^{2+}]_o$ to $[Ca^{2+}]_i$. The restorative effects of KCl were not due to an increase in osmolarity, as substitution of 50 mM NaCl with 50 mM KCl had the same stimulatory activity while addition of 50 mM NaCl instead of KCl had no effect.

Addition of HK^+ to medium containing HCa^{2+} (Fig. 1 *E*) slightly inhibited neurite elongation. On average, neurites grown for 5 d were 76 ± 6% (n = 10) the length of neurites grown in normal medium (Fig. 1, *A* and *E*) and were also shorter than neurites from cultures grown in 50 μM Ca^{2+} and HK^+ (Fig. 1, *G*, *H*, and *E*). The slower rate of outgrowth was apparent throughout the growth period in HK^+ and was not due to an initial delay in neurite extension, suggesting that if the effect of HK^+ were due to an elevation of $[Ca^{2+}]_i$ (21) this effect may have persisted for several days.

A quantitative measure of relative neurite outgrowth was obtained by comparing the extent of uptake of submicromolar doses of [^{3}H]NA. Fig. 2 shows that the amount of

Figure 1. Regulation of neurite outgrowth by $[Ca^{2+}]_o$ and KCl. Explants were cultured for 5 d in L15-CO_2 medium containing 3% rat serum and 19.2 ng/ml NGF. Medium contained 5.4 mM KCl (*A–D*) or 55 mM KCl (*E–H*) and no EGTA (1.3 mM Ca^{2+}; *A* and *E*), 1 mM EGTA (0.3 mM Ca^{2+}; *B* and *F*), 1.1 mM EGTA (150 μM Ca^{2+}; *C* and *G*), or 1.2 mM EGTA (50 μM Ca^{2+}; *D* and *H*). Bar, 400 μm.

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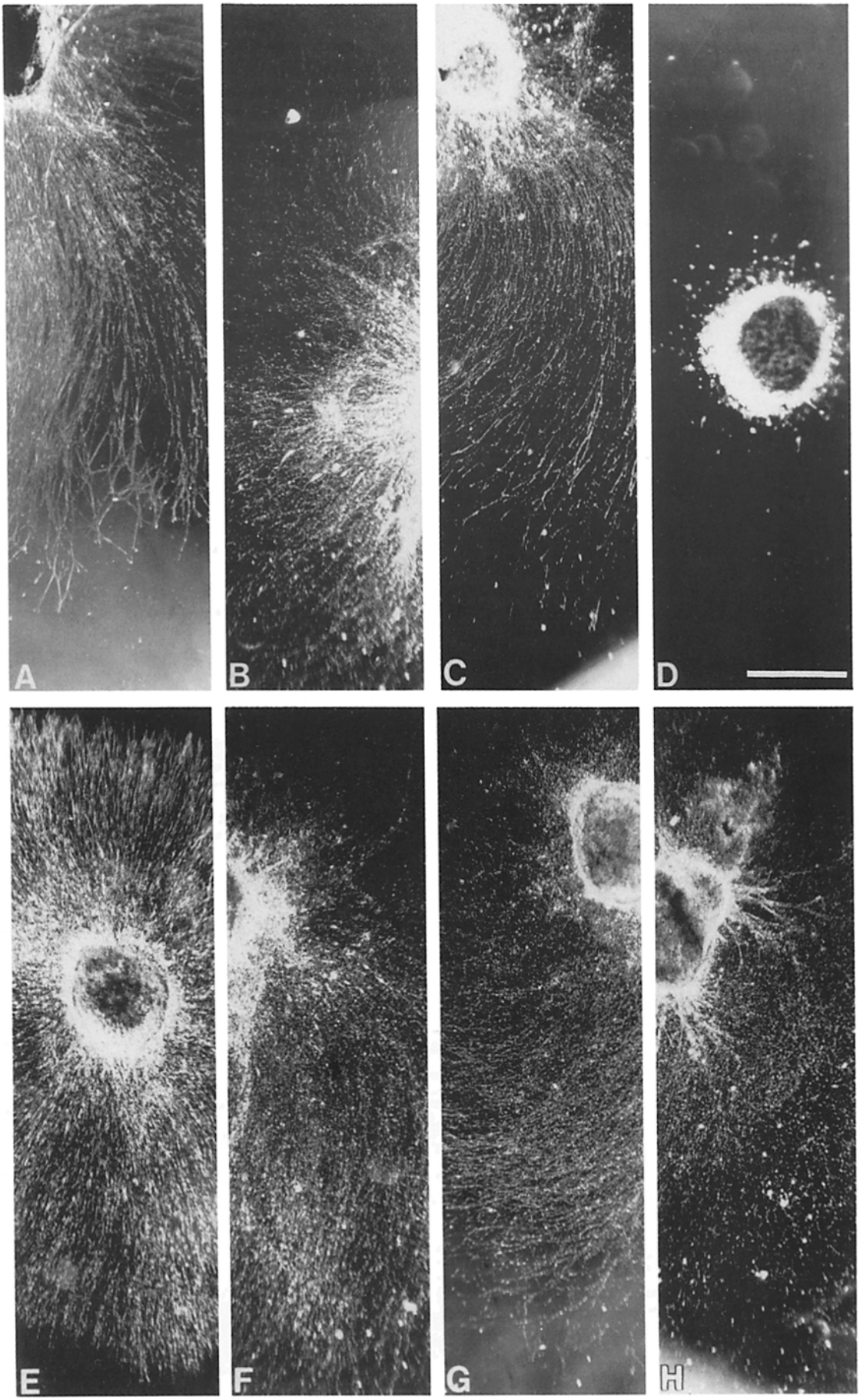

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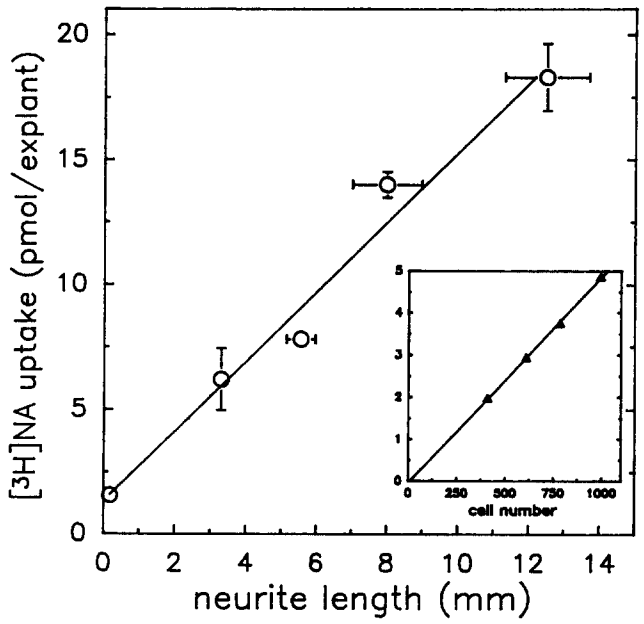

Figure 2. [3H]NA uptake as a function of neurite length and cell number. Explants were cultured for 12 h to 36 d to obtain neurites of various average lengths as indicated on the abscissa. Cultures were washed, incubated with [3H]NA (1.49 μM) for 40 min, and washed again, and total [3H]NA uptake was measured. The insert shows [3H]NA uptake into cultures of single cells (13 d) that were counted before the experiment.

[3H]NA uptake was highly correlated to average neurite length and to cell number, as was previously shown for [3H]adenine uptake (45). Moreover, the amount of [3H]NA taken up by the cell bodies was negligible as compared with neurites: for example, in 23-d explants uptake into neurites was 60 ± 8-fold higher than into cell bodies (n = 3). Furthermore, when neurites were divided into concentric segments with ~0.8-, 1.6-, and 3.5-mm cross sections beginning from the center (44, 45) before [3H]NA uptake, uptake into each segment was proportional to its cross-sectional length (1.6, 2.6, and 6.9 pmol, respectively). Thus, for cultured sympathetic neurons, [3H]NA uptake provides a rapid method to quantitate relative neurite outgrowth. Table I shows the extent of [3H]NA uptake into 10-d explants including those shown in Fig. 1. Total neurite outgrowth was similar between the different culture conditions although the specific density was slightly elevated in those cultures grown in HK media, suggesting that there were more neurites in these cultures.

Three additional architectural features of outgrowth were noted. First, in HK^+/HCa^{2+} medium, neurites appeared to grow more linearly, giving the leading edges of neurites a blunt appearance (Fig. 1 *E*), whereas in the other media outgrowth was less regular (e.g., Fig. 1 *A*). There were no apparent changes in growth cone morphology between the different media when observed under the light microscope. Second, in the HK^+/LCa^{2+} medium, neurites appeared to be thinner. Third, the progressive reduction of $[Ca^{2+}]_o$ decreased neurite fasciculation. These last two effects will be discussed further below (Fig. 6).

Characterization of the Ca^{2+} Channels That Control Ca^{2+} Influx

The membrane potential from cell bodies of isolated cultured neurons grown in normal medium was −66 to −60 mV. In all cells grown in the HK^+ media, or in cells to which 50 mM KCl was added acutely, the membrane potential was depolarized to −15 to −5 mV as measured using a patch pipette in the whole cell recording mode. This depolarization activated both Na^+ and Ca^{2+} channels but also caused them to inactivate (Murrell, R. D., and A. M. Tolkovsky, manuscript in preparation). To determine that activation of voltage-sensitive channels was required for outgrowth in the HK^+/LCa^{2+} medium, the dependence of neurite outgrowth on the extent of depolarization was examined (Fig. 3). As predicted, the average length and number of neurites was directly related to the concentration of KCl added, with half-maximal neurite outgrowth obtained at ~20–25 mM KCl. Below 10–15 mM KCl no neurites were observed. Fig. 3 *B* shows the quantitative relationship between [3H]NA uptake and KCl concentration. The possible involvement of Na^+ channels was excluded by adding 2 μM tetrodotoxin to the various media, which had no effect although this concentration of tetrodotoxin largely abolished the action potential in isolated neurons. Ca^{2+} channel blockers were used to identify the Ca^{2+} channels involved.

Table I. Quantitation of Neurite Outgrowth

	$[Ca^{2+}]_o$	$[KCl]_o$	Length	[3H]NA/uptake	Specific density
	mM	*mM*	*mm/explant*	*pmol/explant*	*pmol[3H]NA/mm*
−Nifedipine	1.3	5	4.2 ± 0.1	4.9 ± 0.9	1.17 ± 0.19
	1.3	50	3.6 ± 0.2	4.8 ± 0.5	1.33 ± 0.11*
	0.15	5	4.0 ± 0.3	4.4 ± 0.6	1.09 ± 0.16
	0.15	50	2.9 ± 0.1	4.0 ± 0.2	1.38 ± 0.06‡
	0.05	50	3.1 ± 0.05	3.9 ± 0.2	1.26 ± 0.07
+Nifedipine	0.15	50	3.4 ± 0.4	4.5 ± 0.3	1.32 ± 0.13
	0.05	50	0.15 ± 0.15	0.02 ± 0.02	0.13 ± 0.19

Explants (including those shown in Figs. 1 and 3) were washed in L15-air medium after 10 d in culture, incubated in the same medium containing 0.34 μM [3H]NA for 30 min at room temperature, and washed as previously described (45). The average length of neurite outgrowth was calculated from measurements of the perimeters of the cultures. The specific density, which normalizes NA uptake to unit length, is a relative measure of the average number of neurites per explant since [3H]NA uptake is directly proportional to cell number and to neurite length. The data represent two separate experiments done in triplicate. A two-tailed t test was used to examine the probability that the specific densities are different between the various cultures.

* $0.2 < \alpha < 0.4$, rows 1 and 2.

‡ $0.02 < \alpha < 0.05$, rows 3 and 4.

§ Nifedipine concentrations were 50 or 1 μM in the presence of 0.15 or 0.05 mM $[Ca^{2+}]_o$, respectively.

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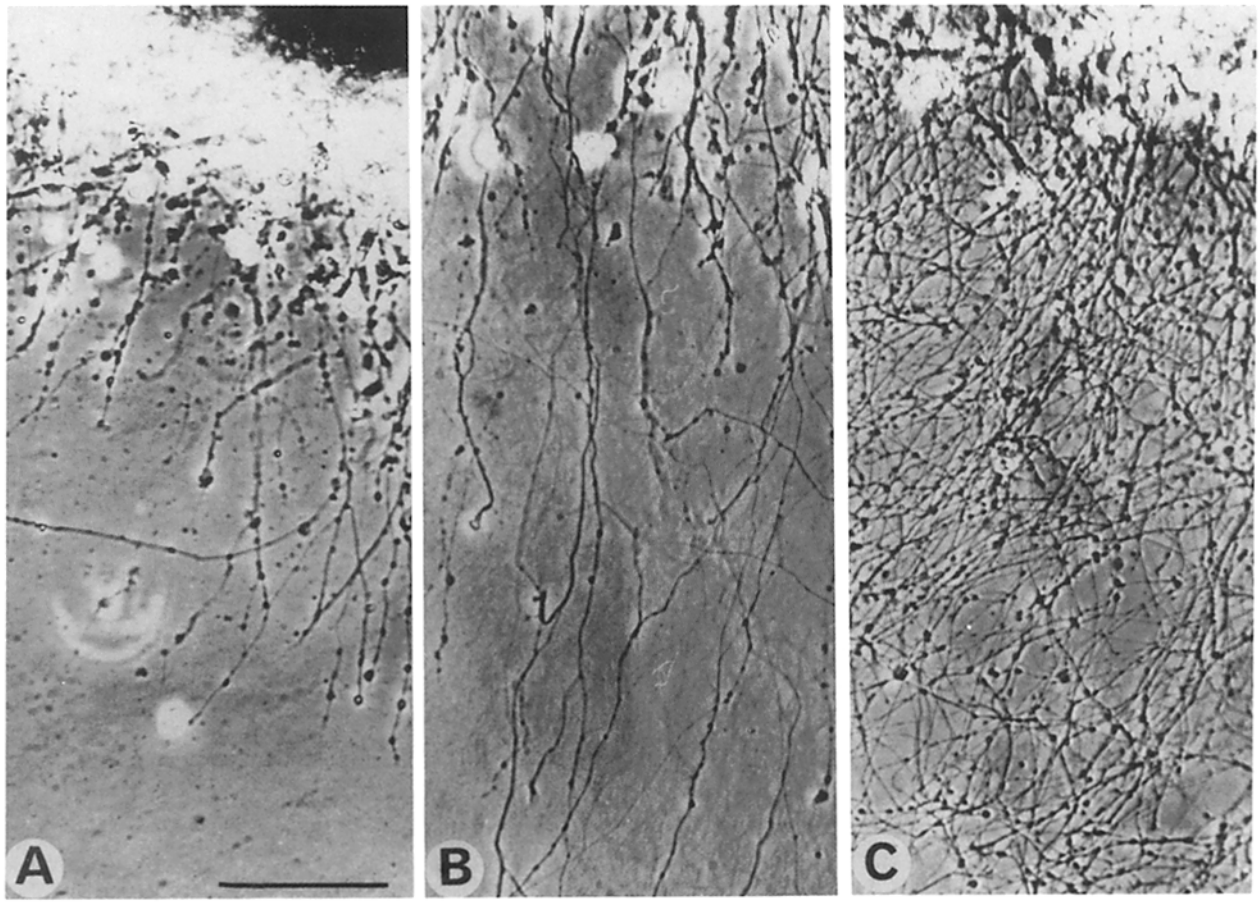

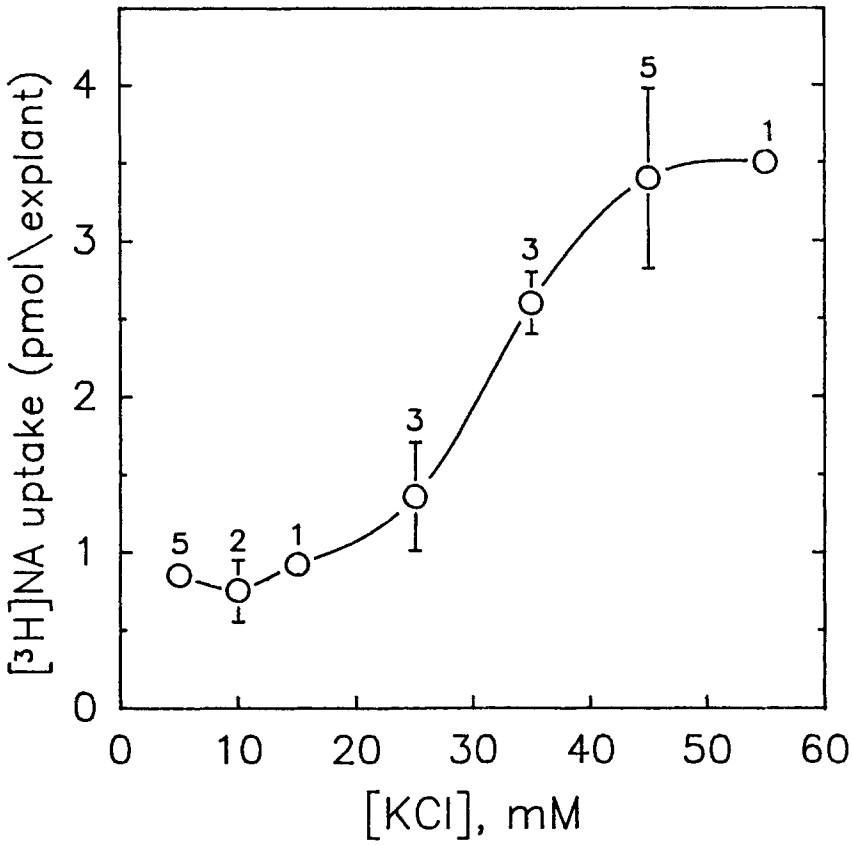

Figure 3. Voltage dependence of neurite outgrowth. Culture medium contained 1.2 mM EGTA (50 μM Ca^{2+}) and 15 mM KCl (*A*), 25 mM KCl (*B*), or 55 mM KCl (*C*). In *A*, the explant can be seen at the top of the photograph; in *B*, the explant is just off the top edge. The longest neurites were ~240 μm, just off the bottom edge. In *C*, the explant is in the top right corner. Neurites extended to 2.5 times the length shown in the photograph, ~530 μm. Bar, 40 μm. (*D*) Explants grown in LCa^{2+} medium (1.2 mM BAPTA) containing 5–55 mM KCl for 6 d were washed and incubated for 1 h in normal medium containing 6 μCi/ml [^{3}H]NA (0.43 μM) to quantitate total neurite outgrowth. Average neurite length was up to 0–0.1, 0.14 ± 0.04, 0.5 ± 0.14, 0.94 ± 0.2, and 1.2 mm for 15, 25, 35, 45, and 55 mM KCl, respectively. A regression analysis of the uptake per length slope gave an $R = 0.92 \pm 0.1$.

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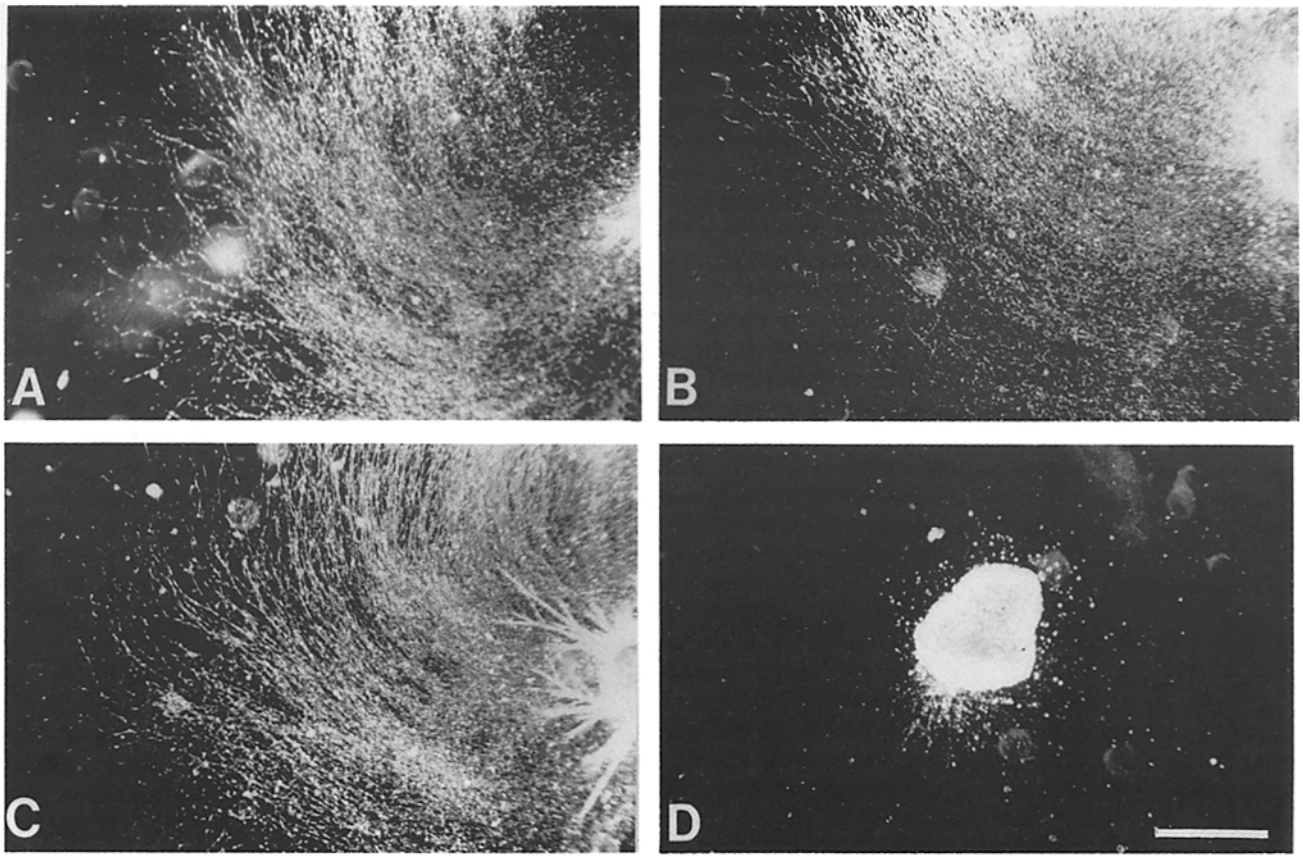

Figure 4. L type Ca^{2+} channels mediate Ca^{2+} influx for neurite outgrowth. Explants were cultured for 5 d in medium containing 55 mM KCl and either 1.1 mM EGTA (150 μM Ca^{2+}) (*A* and *C*) or 1.2 mM EGTA (50 μM Ca^{2+}) (*B* and *D*). Nifedipine was diluted 100-fold from a 10 mM stock in DMSO and was added to explants at the time of plating after further dilution to 50 μM (0.5% DMSO) (*C*) or to 1 μM (*D*). 0.5% DMSO had no effect on neurite outgrowth. Bar, 400 μm.

ω-conotoxin GVIA, which has been reported to block both L and N type Ca^{2+} channels in several neurons (34) but which may not block L type channels in superior cervical ganglion neurons (38), had no effect when added at 1 μM (*a*) at the time of plating, (*b*) when cells were preincubated with ω-conotoxin GVIA before plating, and (*c*) when it was added daily to the culture medium to reduce toxin degradation. In contrast, L type channel antagonists were potent inhibitors of neurite outgrowth when added to cultures grown in HK^+ and 50 μM Ca^{2+} (Fig. 4 and Table I): the 1,4-dihydropyridines (DHPs) nifedipine and nitrendipine blocked at 0.3 μM, methoxyverapamil (D600) blocked at 1 μM, and diltiazem blocked at 5 μM. These drugs potently inhibit L type Ca^{2+} channels through separate molecular sites in heart cells, where the lower potency of diltiazem was also observed (26, 41). DHPs also block L type channels in sympathetic neurons (18). Fig. 4 also shows that the inhibition of outgrowth by nifedipine was not due to nonspecific, hydrophobic, or toxic activity since no effect on growth was observed when $[Ca^{2+}]_o$ was raised from 50 to 150 μM in the HK^+ medium although nifedipine concentration was raised from 1 to 50 μM (Fig. 4 *C*). A higher concentration of nifedipine was used to compensate for the reduction in its apparent affinity to L type channels due to the increase in $[Ca^{2+}]_o$ (4, 26, 41). It was therefore concluded that L type channels mediate the rate-limiting influx of Ca^{2+} into neurons cultured in the HK^+/LCa^{2+} medium.

Measurement of Intracellular Ca^{2+} in Ca^{2+}-clamped Neurons

Since the HK^+/LCa^{2+} medium acted as a vast, Ca^{2+}-buffered reservoir and the open Ca^{2+} channels were freely permeable to Ca^{2+}, $[Ca^{2+}]_i$ should be effectively clamped from outside the cell, regardless of the mechanisms that operate to maintain Ca^{2+} homeostasis (7). To determine the $[Ca^{2+}]$ in the cell bodies that was set under these conditions and to see whether $[Ca^{2+}]_i$ was indeed clamped so that the cells were unable to generate a Ca signal, $[Ca^{2+}]_i$ was measured using a fluorescent Ca^{2+} indicator, fura-2. As growth media were used in these experiments to simulate culture conditions, the effect of NGF and serum on $[Ca^{2+}]_i$ was first examined. Fig. 5 *A* shows that NGF had no effects on $[Ca^{2+}]_i$ although neurons were first depleted of NGF by a 30-h incubation in NGF-free medium. The two 30-min records were flat and nearly superimposed. NGF was also added to NGF-deprived neurons after bradykinin, a protocol used by Van Calker et al. (47), to show that NGF enhances the elevation of $[Ca^{2+}]_i$ promoted by bradykinin in PC12 cells (Fig. 5 *B*). Although bradykinin caused an increase in $[Ca^{2+}]_i$ similar to PC12 cells, NGF had no additional stimulatory effects. Subsequent addition of carbachol (100 μM) and KCl (50 mM) caused further elevations of $[Ca^{2+}]_i$, demonstrating that the cells were still able to respond to agents that increase $[Ca^{2+}]_i$ by opening Ca^{2+} channels and perhaps by releasing

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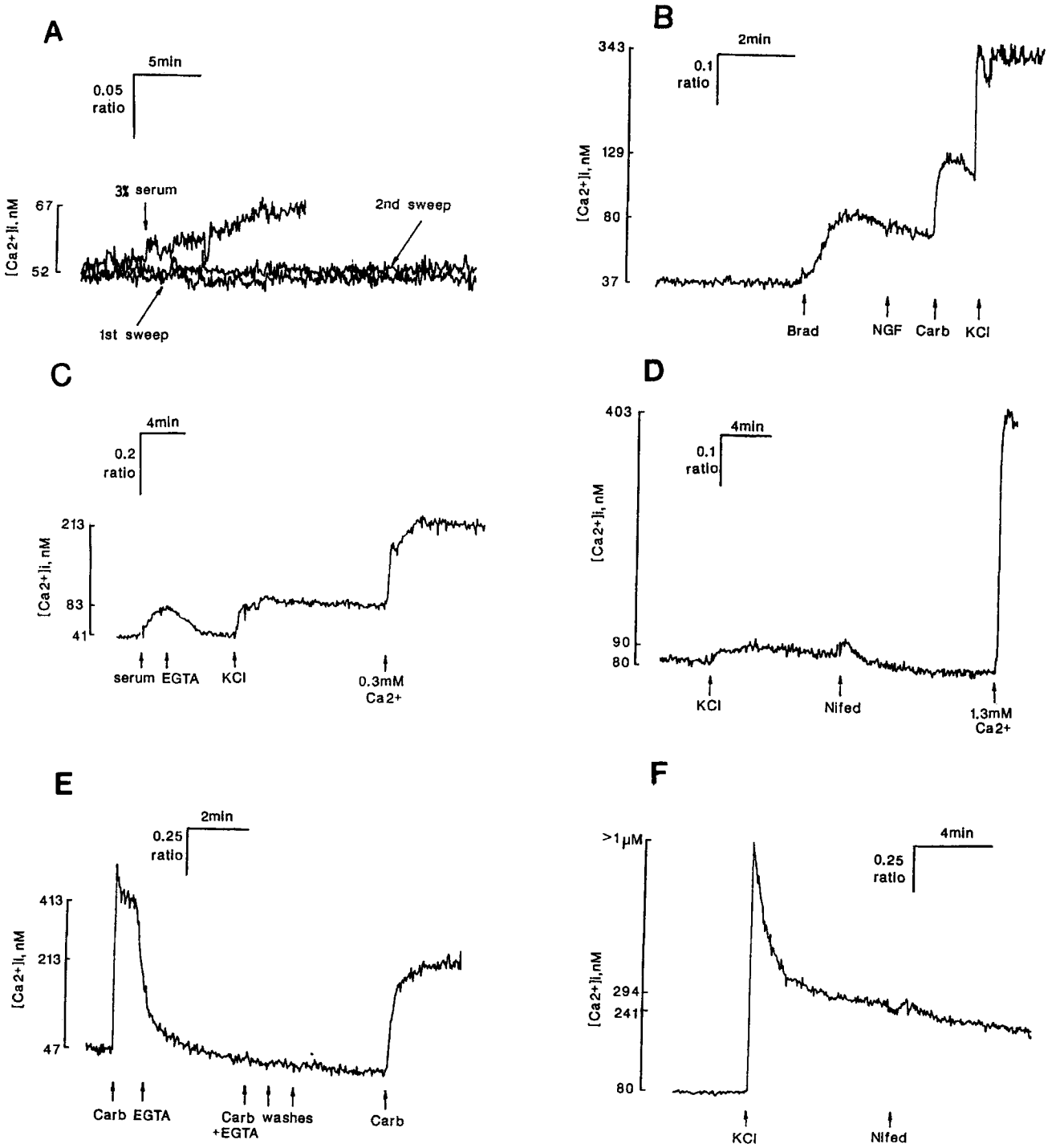

Figure 5. $[Ca^{2+}]_i$ is clamped at resting levels in HK^+/LCa^{2+} medium. (*A*) 192 ng/ml NGF was added to neurons that were deprived of NGF for 30 h. After continuous recording for 65 min, 3% serum was added (13-d cultures, six cell bodies and neurites). (*B*) Bradykinin (*Brad*) (90 nM) was added to neurons deprived of NGF for 24 h followed by 192 ng/ml NGF, 100 μM carbachol (*Carb*), and 50 mM KCl as indicated (13-d cultures, six cell bodies and neurites). (*C*) Neurons were incubated in normal growth medium containing 192 ng/ml NGF; at times indicated 3% serum was added followed by 1.2 mM EGTA (or 50 μM Ca^{2+}), 50 mM KCl, and 300 μM $CaCl_2$ (23-d cultures, three cell bodies and neurites). (*D*) Neurons grown in the HK^+/LCa^{2+} medium were incubated in the same medium lacking KCl; at the times indicated 50 mM KCl was added followed by 2.2 μM nifedipine (added as a ninefold concentrate) and 1.3 mM $CaCl_2$ (12-d cultures, four cell bodies and neurites). (*E*) Carbachol (100 μM) was added to neurons incubated in L15 medium without serum or NGF. EGTA (1.2 mM) was added followed by 100 μM carbachol in 1.2 mM EGTA. Neurons were washed in L15 medium, and 100 μM carbachol was again added (23-d cultures, one cell body). (*F*) 50 mM KCl was added to neurons grown in normal medium; at the arrow 50 μM nifedipine was added as a twofold concentrate (12-d cultures, eight cell bodies and neurites).

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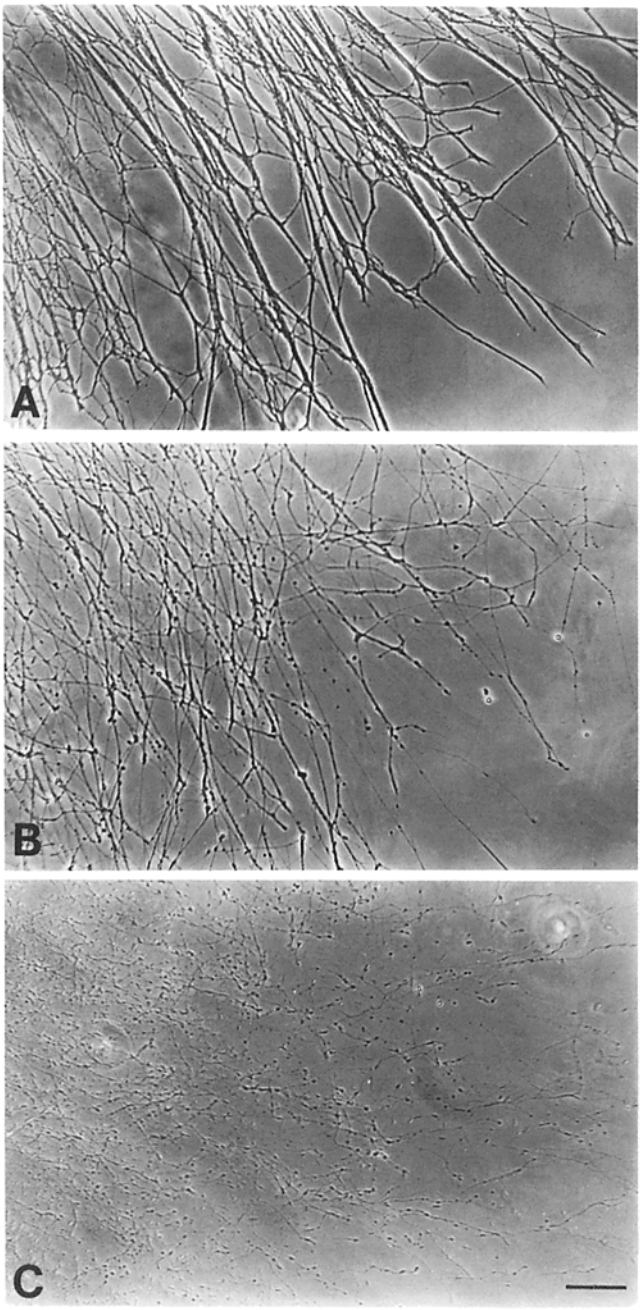

Figure 6. Fasciculation is controlled by extracellular Ca^{2+}. Explants were cultured for 5 d in (*A*) the absence of EGTA, (*B*) in the presence of 1.1 mM EGTA and 55 mM KCl, or (*C*) 1.2 mM EGTA and 55 mM KCl. Bar, 100 μm.

Ca^{2+} from intracellular stores (carbachol; Suidan, H. S., and A. M. Tolkovsky, unpublished observations). In contrast, serum (3%) consistently caused a small but sustained increase in $[Ca^{2+}]_i$ from 44 ± 6.6 to 76 ± 8.5 nM (n = 4) (Fig. 5 *A*, 65 min after NGF; see also Fig. 5, *C*, *D*, and *F*.

Fig. 5 *C* shows that reconstitution of the LCa^{2+} medium with 1.2 mM EGTA (as in Fig. 1 *D*) caused a drop in $[Ca^{2+}]_i$ to the levels observed in the absence of serum. These same conditions were not permissive for growth. The addition of 50 mM KCl, which permitted growth (Fig. 1 *H*), elevated $[Ca^{2+}]_i$ but only back to the resting levels previously set by serum. $[Ca^{2+}]_i$ remained stable at this level over several hours under these conditions (not shown). The stability of $[Ca^{2+}]_i$ is also evident from the observation that four separate cultures of neurons that were grown in the HK^+/LCa^{2+} medium and were loaded with fura-2 in that medium did not report a significant change in resting $[Ca^{2+}]_i$ (not shown). To show that Ca^{2+} channels remained open in the HK^+/LCa^{2+} medium, $[Ca^{2+}]_o$ was raised to 300 μM, and an immediate increase in $[Ca^{2+}]_i$ above resting levels was observed. In the absence of HK^+ but in LCa^{2+}, however, the addition of this amount of Ca^{2+} had little effect on $[Ca^{2+}]_i$. In Fig. 5 *D*, the effect of nifedipine, which blocked neurite outgrowth, is shown. Neurons grown in the HK^+/LCa^{2+} medium were preincubated for 2 h in LCa^{2+} medium in the absence of KCl. The addition of 50 mM KCl caused a small increase in $[Ca^{2+}]_i$ that was reversed by nifedipine (2.2 μM) down to levels seen before the addition of KCl. We estimate that the changes in $[Ca^{2+}]_i$ that permitted or blocked neurite outgrowth were not < 30 nM. The suggestion that Ca^{2+} channels remained open and functional in the HK^+/LCa^{2+} medium for long periods is also supported by the observation that withdrawal of KCl caused an immediate cessation of neurite outgrowth. The effect of adding an agonist that elevates $[Ca^{2+}]_i$ in normal medium to neurons under clamp conditions was also examined (Fig. 5 *E*). Although carbachol rapidly raised $[Ca^{2+}]_i$ in neurons incubated in normal medium, under the Ca^{2+} clamp no such rise in $[Ca^{2+}]_i$ was obtained. This was not due to a desensitization of the receptor since another response to carbachol was elicited rapidly after restoring normal $[Ca^{2+}]_o$. The responses shown are from a single cell.

The effect of adding HK^+ to neurons grown in normal medium (conditions as in Fig. 1 *E*) is also shown (Fig. 5 *F*). After a rapid increase, $[Ca^{2+}]_i$ declined to a constant level of ~300 nM, well above resting levels. Nifedipine (50 μM) reduced $[Ca^{2+}]_i$ only slightly, suggesting that most of the persistent rise in $[Ca^{2+}]_i$ was not maintained by supply of Ca^{2+} through L type voltage-dependent channels.

Fasciculation and Neurite Outgrowth

A progressive loss of fasciculation with decreasing $[Ca^{2+}]_o$ was observed; shown in detail in Fig. 6. This effect was directly related to $[Ca^{2+}]_o$ since $[Ca^{2+}]_i$ was the same in LK^+ media containing 1.3 mM to 150 μM $[Ca^{2+}]_o$ or in the HK^+/LCa^{2+} medium. There was no clear correlation between the degree of fasciculation and the rate or extent of neurite elongation (compare with Fig. 1, *A*, *C*, *G*, and *H*). The distribution of neural cell adhesion molecule (N-CAM), which has been suggested to be a major determinant of fasciculation in NGF-responsive chick spinal ganglion neurons (39), was also not affected (Fig. 7). N-CAM immunoreactivity was highest at attachment sites between neurites and the substratum when the antibody was reacted for 30 min with live neurons at 23°C. Three varicosities are magnified (Fig. 7 *C*) to show the adhesion points. To examine whether the loss of fasciculation was due to the reduction in divalent ion concentration, $MgCl_2$ (10–30 mM) was added to the normal medium to displace Ca^{2+}. Although fasciculation was blocked almost completely, there was no effect on the extent of neurite outgrowth or on $[Ca^{2+}]_i$ (not shown).

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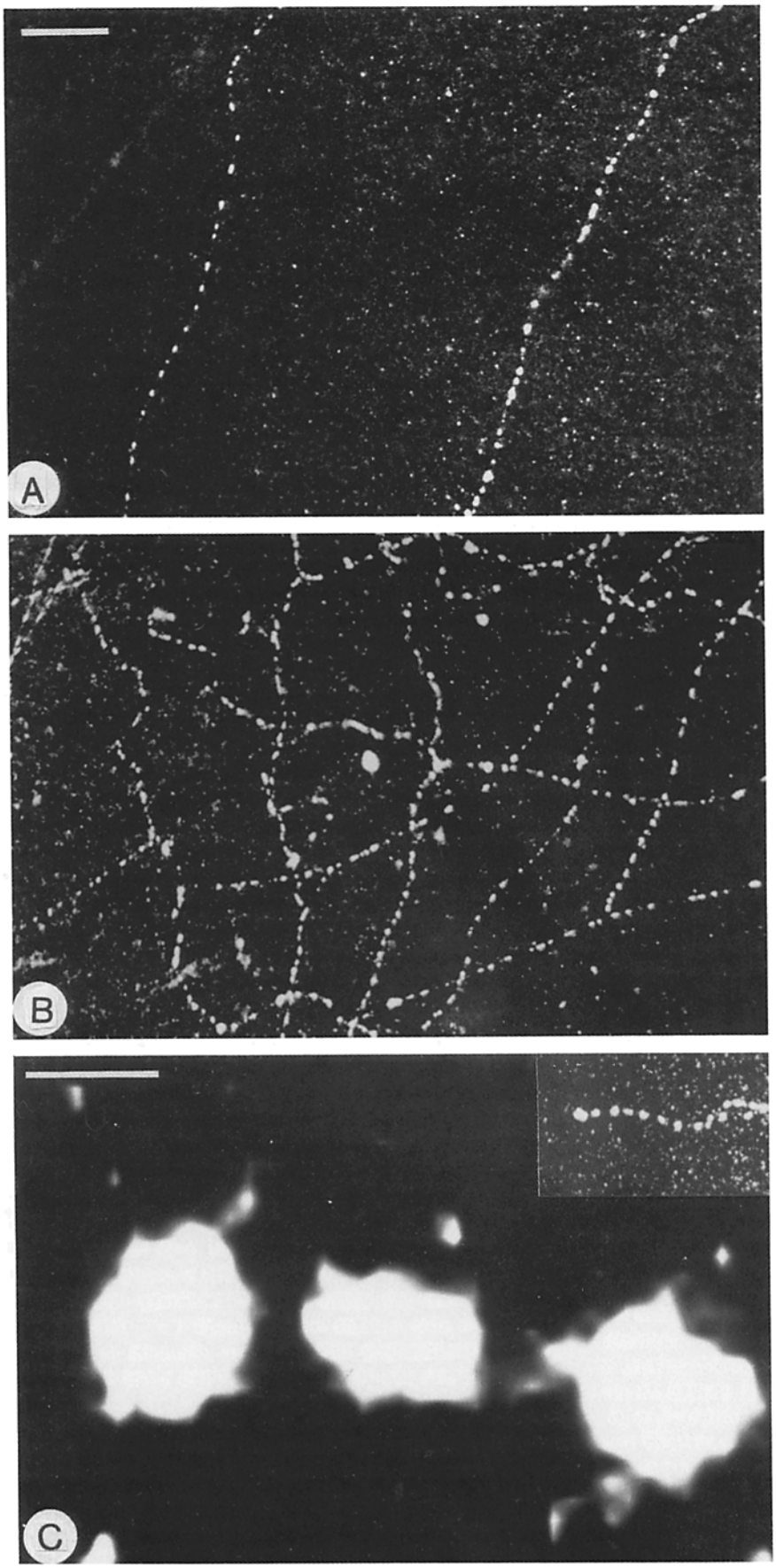

Figure 7. The anti–N-CAM polyclonal antibody C31/3 was used to visualize N-CAM on living cultures of neurons grown in (*A*) normal medium or (*B*) HK^+/LCa^{2+} medium. (*C*) a detail of three varicosities shown in the insert is magnified. The anti–N-CAM antibody (C31/3) was raised by Dr. Christine Booth (Department of Physiology, University of Oxford) against affinity-purified N-CAM from mouse brain. The antibody was diluted 1:250 into L15 medium containing 5% horse serum (Gibco Laboratories), and 100 μl was added per culture. After 1 h at room temperature, coverslips were washed extensively in PBS and incubated with FITC-conjugated donkey anti-rabbit Ig (Amersham Corp.) diluted 1:20 in the same medium. After 30 min at room temperature, coverslips were washed in PBS and mounted in Gelvatol. Cells were visualized using an MRC-500 confocal laser microscope (Bio-Rad Laboratories) and a 60× objective. Images were averaged over 25–34 sweeps. No fluorescence was detected on neurites in the absence of the first antibody. Bars: (*A* and *B*) 25 μm; (*C*) 25 μm.

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Table II. Uptake and Release of [^{3}H]NA by Explants and Single Cell Cultures Grown in HCa^{2+} or LCa^{2+} Media

	$[Ca^{2+}]_o$	[KCl]	[^{3}H]NA uptake	[^{3}H]NA released	
				$BaCl_2$	$CaCl_2$
	mM	*mM*	*pmol*	%	%
Explants	1.3	5	8.7 ± 2.5	20.3 ± 1.8	2.9 ± 0.6
	0.05	50	10.4 ± 2.5	25.7 ± 3.2	2.8 ± 0.8
Single cells	1.3	5	7.7 ± 2.3		9.9 ± 1.5
	1.3	50	8.7 ± 2.7		8.1 ± 0.1
	0.05	50	11.1 ± 2.0		8.2 ± 1.6

Explants cultured in normal medium or in HK^+/LCa^{2+} medium for 5 d were incubated for 1 h at 37°C in an L15-air medium containing 5 μCi/ml (0.34 μM) [^{3}H]NA (15 Ci/mmol). Release was induced for 2 min with 55 mM KCl and 3 mM $BaCl_2$ (n = 3) or 1.3 mM $CaCl_2$ (n = 4) after a series of control incubations. The method of sampling of incubation media was as described previously (45). Basal release was constant and comprised 0.5% of the total uptake per 2-min period. The average lengths of neurites after 110 h in normal and in HK^+/LCa^{2+} media were 1,520 ± 330 μm (n = 5) and 1,320 ± 280 μm (n = 4), respectively. Single cells cultured in 24-well multiwells for 12 d in normal, HK/HCa^{2+}, and HK/LCa^{2+} (buffered with 1.2 mM BAPTA) media were washed and incubated for 1.5 h at 37°C in normal medium containing 2.2 μM [^{3}H]NA (15 Ci/mmol). Duplicate samples were washed in L15 medium at room temperature using suction under vacuum, and release was initiated using an L15 salt solution containing 50 mM KCl. Remaining radioactivity was extracted with 5% aqueous acetic acid to calculate percent release. Basal release was 2.2 ± 0.5% of total uptake over a 4-min period (n = 6).

[^{3}H]NA Release

Despite the differences in the pattern of fasciculation, and the apparently thinner neurites, cultures grown in HK^+/LCa^{2+} or in normal medium took up (Tables I and II) and released similar amounts of the neurotransmitter [^{3}H]NA (Table II). The ability of neurons grown in HK^+/LCa^{2+} medium to release [^{3}H]NA was also retained when explants were transferred to normal medium containing HK^+ or to a solution containing 3 mM $BaCl_2$ and HK^+ (Table II), but no release was obtained in the HK^+/LCa^{2+} medium itself. The ability of the low Ca^{2+} medium to sustain survival and growth but not to support neurotransmitter release, even when the medium was first changed to a nondepolarizing medium to remove inactivation of VSCC (not shown), extends the results obtained with fura-2 by suggesting that $[Ca^{2+}]_i$ in the neurites was indeed kept clamped within very narrow limits.

Discussion

This study shows that several millimeters of neurite outgrowth from sympathetic neurons cultured for several days can be obtained when intracellular Ca^{2+} levels are prevented from rising above resting levels. Thus, although it may be possible that the resting level of Ca^{2+} varies in different regions of the cell so that there is a gradient of Ca^{2+} concentrations between the cell bodies and the growth cones, a Ca^{2+} signal is not a prerequisite for normal neurite extension. It is also highly unlikely that elevation of $[Ca^{2+}]_i$ is involved in the actions of NGF since the presence of NGF was necessary for survival and growth throughout the culture period, long after $[Ca^{2+}]_i$ was permanently clamped at a constant low level. Extracellular Ca^{2+} was important for promoting fasciculation, but even in the absence of fasciculation normal rates of neurite extension occur and similar amounts of neurites are expressed as in normal medium.

Ca^{2+} Influx during Sustained Depolarization Is through L Type Channels

To establish the Ca^{2+} requirement for long-term growth, a method was sought that would clamp $[Ca^{2+}]_i$ at low levels for several days. This required direct control over $[Ca^{2+}]_i$ from outside the cell, a condition achieved by restricting Ca^{2+} influx to VSCC. By depolarizing the cells in medium containing a maximum of 50 μM $[Ca^{2+}]_o$, neurite outgrowth became dependent on the extent of depolarization (Fig. 2) and was completely blocked by L type Ca^{2+} channel antagonists (Fig. 3).

Although it has been suggested that superior cervical ganglion neurons contain 80–85% N type Ca^{2+} channels and only 10–15% L type Ca^{2+} channels (38), it is clear from the ability of all three classes of L type Ca^{2+} channel antagonists (the DHPs, D600, and diltiazem) to block neurite outgrowth that the L type channels were the only type of VSCC mediating the Ca^{2+} influx required for long-term growth in HK^+/LCa^{2+} medium. The ability of L type channels to mediate Ca^{2+} influx that can be used for growth contrasts with the inability of L type channel-mediated Ca^{2+} influx to release [^{3}H]NA during depolarization, even in 300 μM $[Ca^{2+}]_o$ (18). Recently, Koike et al. (23) have shown that DHPs, but not verapamil, also block the survival-promoting effects of HK on sympathetic neurons from which NGF had been withdrawn for 2 d. Hirning et al. (18) have suggested that the difference between the Ca^{2+} influx mediated by the two channel types lies in the microscopic sites of Ca^{2+} rise and their relation to the cellular machinery for release. Thus, L channels may transmit Ca^{2+} away from the plasma membrane. Alternatively, during the much longer time course of events followed here, Ca^{2+} would have time to diffuse to its sites of action from any other site in the cell, especially under maintained depolarization (40). It is less clear why there was no Ca^{2+} influx via N type channels, especially since Plummer et al. (38) have shown that N channels can sustain a macroscopic current at depolarized holding potentials. That activatable N channels are present despite the long-term depolarization is suggested by the ability to sustain neurotransmitter release in cells that were put into 1.3 mM $[Ca^{2+}]_o$ and 50 mM KCl directly from the growth medium containing HK^+/LCa^{2+} (if indeed only N type channel-mediated Ca^{2+} influx is used for neurotransmitter release). The biological functions of N type channels are still poorly understood. However, it is well established that the amount of Ca^{2+} influx through L type channels is dependent on the presence of a Ca^{2+} binding site in the channel. Perhaps N type channels have a similar binding site but with low affinity for Ca^{2+} such that at 50 μM, Ca^{2+} is unable to permeate the channel. The dose-dependent Ca^{2+} permeability through open VSCC can also explain why, although Ca^{2+} influx was occurring continuously through open channels in the HK^+/LCa^{2+} medium, $[Ca^{2+}]_i$ remained 200-fold lower than $[Ca^{2+}]_o$.

Proof of the Ca^{2+} Clamp

The minimal concentration of $[Ca^{2+}]_i$ that permits normal neurite outgrowth or minimal Ca^{2+} set point (32, 33) has been defined here empirically (by titration with increasing concentrations of KCl in LCa^{2+} medium) and, interestingly, it appears to coincide with the normal resting level of Ca^{2+}. This amount of Ca^{2+} is just sufficient for survival since the

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removal of 55 mM KCl or the addition of L type channel blockers at any time during growth caused neurons to degenerate. With the permeability of the membrane to Ca^{2+} maintained at a set level under HK^+/LCa^{2+} conditions, the only way in which a cell could generate a Ca^{2+} signal is through release of Ca^{2+} from intracellular stores. It is unlikely that after such extensive depolarization in LCa^{2+} medium there are sufficient stores to generate a signal. This is supported by the observation that bradykinin, which raises $[Ca^{2+}]_i$ and promotes neurotransmitter release in normal medium (Suidan, H., unpublished observation), could not elevate $[Ca^{2+}]_i$ or cause neurotransmitter release in HK^+/LCa^{2+} medium. In addition, Thayer et al. (43) have shown that rat sympathetic neurons contain relatively small intracellular Ca^{2+} stores. The fact that L type channels remained active throughout the growth period also strongly suggests that $[Ca^{2+}]_i$ remained constant and at a low level during this time, as these would be expected to inactivate if $[Ca^{2+}]_i$ increased above a certain threshold (26). Thus, it is unlikely that an increase in $[Ca^{2+}]_i$ beyond resting levels is required for neurite outgrowth or for the survival-promoting actions of NGF.

The sharp transition between block and resumption of outgrowth after small changes in $[Ca^{2+}]_i$ from ~70 to ~40 nM in the presence of open VSCC suggests that there is a metabolic rate-limiting process that is highly Ca^{2+} sensitive. This is likely to be protein synthesis (10, 11). There may have been a slight reduction in protein synthesis even in the HK^+/LCa^{2+} medium as neurites appeared slightly thinner than those grown in normal medium. Koike (22) also reported that neurites grown from PC12 cells in 10 μM Ca^{2+} were ~66% the thickness of neurites grown in 10 mM Ca^{2+}, and Brostrom et al. (10) have shown that reduction of $[Ca^{2+}]_o$ to ~10 μM reduces protein synthesis in PC12 cells by 50%. Walicke and Patterson (48) have shown that neurons that survived in conditioned medium containing EGTA were not altered in their capacity to synthesize NA from tyrosine but had increased amounts of choline acetyltransferase activity. As neurite elongation and the total capacity to store and release NA were not altered in explants grown in HK^+/LCa^{2+} medium, it will be interesting to examine whether there are specific classes of proteins that are altered under the Ca^{2+} clamp conditions. DHP binding studies are under way to see whether L type channel number or distribution is changed.

Neurite Fasciculation and Substrate Adhesion

It is clear from the present experiments that extracellular Ca^{2+} is of major importance in promoting adhesion between neurites. Although there was little difference in the amount of N-CAM on neurites grown in low or high Ca^{2+}, N-CAM may be important for adhesion between the neurites and the substrate since it was highly concentrated at varicosities that adhered to the substratum. The spacing of these varicosities is similar to kinesin-immunoreactive varicosities (37), but Pfister et al. (37) suggest that these varicosities are similar to the mobile "parcels" described by Hollenbeck and Bray (19). Aletta and Greene (1) have also described parcels that are produced from growth cones of sympathetic neurons grown on collagen. In contrast to these parcels, the N-CAM–rich structures shown here are immobile and are also evenly spaced. Thus, although neurites are still growing, they are also firmly attached to the substrate. If, as has been suggested (8, 24), growth cones exert tension to control elongation, this would be transmitted only as far back as the next bouton. It is therefore difficult to see how cell bodies would receive information about this tension to regulate neurite elongation.

Growth in the HK^+/HCa^{2+} Medium

The linear pattern of outgrowth in the HK^+/HCa^{2+} medium is reminiscent of the smooth patterns observed when neurons are treated with actin-disrupting agents (5, 30). Lankford and Letourneau recently reported (25) that one of the short-term effects of raising $[Ca^{2+}]_i$ in chick dorsal root ganglia was a disruption of peripheral actin filament assembly. However, they showed that this caused a retraction of neurites and a decrease in filopodial movement followed by cessation of neurite growth similar to Campenot (12). This contrasts with the long-term effects observed in this study, where cells grown in HK^+/HCa^{2+} medium had neurites that were ~80% the length of those growing in normal medium. Perhaps most of the normal neurite elongation in these cultures is determined by microtubule assembly and not by growth cone activity (see also Mitchison and Kirschner [35]). The role of growth cones as pathfinders in these cultures would be expected to be marginal, considering the chemically uniform and adhesive nature of the substratum. Indeed, these growth cones are generally poor in spikes and lamellipodia. It remains to be seen if there are subtler changes in growth cone morphology, and the effects of actin-disrupting drugs need to be examined before further conclusions about the role of growth cones in promoting long-term neurite elongation in these cultures can be made.

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